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Effect of Chronic Exposure to Aluminium on Isoform Expression and Activity of Rat (Na^+/K^+)ATPase

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The ability of aluminum to inhibit the (Na^+/K^+)ATPase activity has been observed by several investigators. The (Na^+/K^+)ATPase is characterized by a complex molecular heterogeneity that results from the expression and differential association of multiple isoforms of both catalytic (α) and regulatory (β) subunits. For instance, three main α (α_1, α_2 and α_3) and three β (β_1, β_2 and β_3) subunit isoforms exist in vertebrate nervous tissue, whereas only α_1 and β_1 have been identified in kidney. However, no studies have focused on determining the change in (Na^+/K^+)ATPase isoforms caused by chronic exposure to aluminum and its relation with aluminum toxicity. In this study, adult male Wistar rats were submitted to chronic dietary $AlCl_3$ exposure (0.03 g/day of $AlCl_3$ for 4 months), and the activity and protein expression of (Na^+/K^+)ATPase isozymes were studied in brain cortex synaptosomes and in kidney homogenates. The intracellular levels of adenine nucleotides, plasma membrane integrity, and aluminum accumulation were also studied in brain synaptosomes. Aluminum accumulation upon chronic dietary $AlCl_3$ administration significantly decreased the (Na^+/K^+)ATPase activity measured in the presence of nonlimiting Mg-ATP concentrations, without compromising protein expression of α-subunit isoforms in brain and kidney. Aluminum-induced synaptosomal (Na^+/K^+)ATPase inhibition was due to a reduction in the activity of isozymes containing α_1-α_2 and α_3-subunits. The onset of enzyme inhibition was accompanied by a decrease of the (Na^+/K^+)ATPase sensitivity to submicromolar concentrations of ouabain, and it preceded major damage in plasma membrane integrity and energy supply, as revealed by the analysis of lactate dehydrogenase leakage and endogenous adenine nucleotides. The data suggest that, during chronic dietary exposure to $AlCl_3$, brain (Na^+/K^+)ATPase activity drops, even if no significant alterations of catalytic subunit protein expression, cellular energy depletion, and changes in cell membrane integrity are observed. Implications regarding underlying mechanisms of aluminum neurotoxicity are discussed.

Key Words: **Aluminum; (Na^+/K^+)ATPase; ouabain; brain; kidney; rat.**

The geochemical processes controlling the cycling and availability of aluminum are significantly modified under the influence of anthropogenic activity. These changes are well documented in the literature (see Exley, 2003 for a recent review), whereas the impact of increased bioavailability of aluminum in the biosphere are not as well known. Human health problems resulting from aluminum exposure are based mainly on acute aluminum poisoning, amyotrophic lateral sclerosis/Parkinson–dementia complex of Guam, renal osteodystrophy, anemia (microcytic, hypochromic), and dialysis encephalopathy produced by uptake of aluminum in excess in people treated with hemodialysis for renal disease. Three organ systems are clearly implicated in the toxic effects of aluminum: bone, the hematopoietic system, and the nervous system. Within the nervous system, aluminum is associated with morphological and biochemical changes that tend to reduce nerve synapses and conduction, promoting neurotoxicity (Yokel, 2000; Yokel *et al.*, 2001).

The effects of aluminum on brain gene transcription have been investigated (Lukiw *et al.*, 1998). Aluminum appears to be localized in the chromatin region of nuclei, where relatively strong interaction between Al(III) and DNA occurs (Wu *et al.*, 2005). Moreover, aluminum compounds are capable of inducing a significant increase in cytogenetic damage, and it is recognized that aluminum interrupts peptide synthesis (Lukiw *et al.*, 1998). Nevertheless, there are few indications of a causal relationship between aluminum-induced genetic damage and the appearance of neurotoxicity.

One of the mechanisms of neuronal destruction in degenerative brain damage is apoptosis, a regulated process of cell death with characteristic morphological changes that include nuclear condensation and fragmentation, DNA damage, cell shrinkage, membrane blebbing, and the formation of membrane-bound apoptotic bodies. Aluminum compounds have been shown to induce neurodegeneration and apoptotic effects in several experimental models (Johnson *et al.*, 2005; Savory *et al.*, 2003). Several studies have indicated that oxidative stress, release of calcium from intracellular stores,

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and perturbation of mitochondrial function may represent important steps in the mechanisms underlying neuronal cell death induced by aluminum (Johnson *et al.*, 2005; Savory *et al.*, 2003). Furthermore, the mechanisms by which aluminum interacts with apoptotic pathways are only partially understood.

The (Na^+/K^+)ATPase (EC 3.6.3.9) is the largest protein complex in the family of P-type ATPases expressed in all living organisms. It is essential for the generation and maintenance of Na^+ and K^+ gradients between the intracellular and extracellular milieux, a prerequisite for basic cellular homeostasis and for functions of specialized tissues. The (Na^+/K^+)ATPase consists of two obligatory subunits, the catalytic α subunit and a regulatory glycoprotein (β subunit), and a third non-obligatory proteolipid component belonging to the FXYD gene family of small ion-transport regulators. Individual genes of at least four α-subunit isoforms and three β-subunit isoforms of (Na^+/K^+)ATPase have been identified in mammals (Serluca *et al.*, 2001). Appropriate enzyme expression and activity adapted to changing physiological demands are assured by a variety of regulatory mechanisms for post-translational modification and short-term regulation and by complex regulation of the expression of isozymes (Blanco and Mercer, 1998; Cornelius and Mahmmoud, 2003; Geering, 2001; Jorgensen *et al.*, 2003; Kaplan, 2002; Mobasheri *et al.*, 2000). For example, in neurons where three α-subunit isoforms are present, during rest conditions the basal ionic gradients are maintained mainly by isozymes containing the α_1- and α_2-subunits, whereas upon depolarization and repeated firing of action potentials those isozymes labor at saturation and maximal activation of α_3, assuring the restoration of the resting membrane potential.

Failure of the (Na^+/K^+)ATPase has been implicated in the pathophysiology of neurodegenerative diseases. Yu (2003) has summarized the critical role of (Na^+/K^+)ATPase in signal transduction and cell death pathways (apoptosis, necrosis, and hybrid cell death). Furthermore, the precise sequences of events at the cellular and subcellular levels that follow failure of (Na^+/K^+)ATPase remain to be identified *in vivo*. Recent studies have demonstrated that tissue-specific (Na^+/K^+)ATPase inhibition plays a fundamental role in apoptosis, and they also suggested that inhibition of this enzyme activity may directly trigger cell death or markedly increase cell susceptibility to other apoptotic insults (Xie and Cai, 2003; Yu, 2003).

The inhibitory effect of *in vivo* and *in vitro* exposure to aluminum on (Na^+/K^+)ATPase activity has been observed by several investigators (Caspers *et al.*, 1994; King *et al.*, 1983; Lai *et al.*, 1980; Lal *et al.*, 1993; Rao, 1992; Sarin *et al.*, 1997; Silva and Gonçalves, 2003). However, no studies have analyzed the changes in the (Na^+/K^+)ATPase isoforms that result from chronic aluminium exposure. Thus in the present study we determined whether *in vivo* exposure to aluminum was associated with specific changes in activity and protein expression of membrane-bound (Na^+/K^+)ATPase isozymes.

MATERIALS AND METHODS

Materials. The primary polyclonal antibodies anti-NASE, anti-HERED, anti-TED were kindly offered by Dr. Thomas Pressley from Texas Tech University, Health Sciences Center. Secondary antibodies were obtained from Chemicon International and from Amersham Biosciences (Little Chalfont, UK). The protease inhibitor cocktail (chymostatin, pepstatin A, leupeptin, and antipain), the Kit Enzyline LDH/HBDH Optimisé Unitaire and the chemi-fluorescence system were purchased from Sigma Chemical Co. (St. Louis, MO), bioMérieux (Carnaxide, Portugal), and Amersham Biosciences (Buckinghamshire, UK), respectively. Amersham Biosciences (Little Chalfont, UK) was the source of Polyvinylidene difluoride (PVDF) Hybond-P membranes. All other reagents were of analytical grade.

Chronic exposure to aluminium chloride. One-month-old male Wistar rats (weight ~250 g) were obtained from Harlan Interfauna Ibérica, S. L., and housed individually in standard laboratory cages in accordance with the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes. The environment was maintained at a temperature of 20° ± 1°C and a humidity of 50 ± 10%. Animals ($n = 10$) were randomly assigned to be chronically exposure to aluminum. $AlCl_3$ (0.03 g) was diluted in a small volume of deionized water and added to cheese curdled with lemon juice just before administration, to ensure the voluntary and rapid ingestion of the total dose. This regime results in exposure to 3.60 g of $AlCl_3$, orally administrated as 120 doses (once daily) over 4 months. Control animals ($n = 10$) received cheese curdled with lemon juice for the duration of the treatment. Food and water were provided *ad libitum* to both animal groups until sacrifice. Food and beverages represent the most common route of exposure to aluminum for the general population. The amount of $AlCl_3$ to be administered was established according to previously reported values of oral uptake that promote neurotoxicity.

Tissue preparation and cellular fractionation. All steps were carried out at 4°C. The collected organs were rapidly removed, chopped, and homogenized in a tenfold volume of ice-cold 320 mM sucrose and 10 mM HEPES-Tris at pH 7.4. Synaptosomal fractions were prepared from brain cortex homogenates by differential centrifugation as described elsewhere (Silva and Gonçalves, 2003). The final pellet was resuspended in 0.32 M sucrose, 10 mM HEPES-Tris (pH 7.4) at a final concentration of ~8 mg protein/ml, as determined by the Biuret method. The samples were frozen in liquid nitrogen and maintained at −80°C until used.

Aluminium quantification. The samples were prepared by chemical precipitation of the organic matter by the addition of ice-cold 0.2 N HNO_3 and 8% trichloroacetic acid (TCA), followed by centrifugation at 3000 rpm for 10 min. The resulting supernatants were used for the determination of aluminum concentrations at 309.3 nm in an atomic absorption spectrometer equipped with a graphite furnace (PerkinElmer 4100-VL) with auto-sampler AS-70. The aluminum concentrations in the samples are expressed as ng Al^{3+}/mg protein.

Western blot analysis. Western blot assays were performed as described previously (Pressley, 1992). Protein samples were pre-treated with a lysis buffer (1 mM EDTA, 1 mM EGTA, 2 mM $MgCl_2$, 25 mM HEPES, pH 7.5) supplemented with 100 μM phenylmethanesulfonyl fluoride (PMSF), 2 mM 1,4-dithiothreitol (DTT), and 1:1000 of a mixture of protease inhibitors, containing chymostatin, pepstatin A, leupeptin, and antipain (1 mg/ml). Then, the samples were mixed (1:1) with SDS-loading buffer (6% SDS, 20% glycerol, 5 mM EDTA, 0.25 M Tris-HCl, at pH 6.8, and 0.02% of bromophenol blue). Samples were heated at 60°C for 5 min, centrifuged (14,000 × *g* for 10 min, at room temperature), and electrophoresed on a 7.5% sodium dodecyl sulfate (SDS)–polyacrylamide gel. Samples were transferred to PVDF membranes at 0.75 A for 90 min, at 4°C. The membranes were washed and incubated with TBS-T (0.1% Tween, 150 mM NaCl, and 25 mM Tris–HCl at pH 7.6), supplemented with 5% (w/v) skim dry milk (M-TBS-T) for 2 h to block nonspecific antibody binding. The membranes were incubated with antibodies

directed against the α_1-, α_2-, and α_3-subunit isoforms of the (Na^+/K^+)ATPase, respectively, using the rabbit polyclonal antibodies, anti-NASE (1:1,000), anti-HERED (1:500), and anti-TED (1:1,000) in 0.5% (w/v) M-TBS-T overnight at 4°C. The antibodies were raised against NASE (KNPNASEPKHLL), HERED (KHEREDSPQSHVL), and TED (KHETEDPNDNRYL) sequences of the rat (Na^+/K^+)ATPase α_1-, α_2-, and α_3-subunits, respectively (Pressley, 1992). Actin expression was used as a loading control by incubating membranes with a mouse anti-α-actin monoclonal antibody (1:500) for 2 h at room temperature in 0.5% (w/v) M-TBS-T. The membranes were incubated with anti-rabbit and anti-mouse IgG secondary antibodies (1:20,000), for 2 h at room temperature with gentle shaking, and developed using ECF fluorescence reagent. Immunoreactive bands were visualized by the VersaDoc Imaging System (Bio-Rad, Hercules, CA). The fluorescence signal was analyzed with the QuantityOne software.

Assessment of lactate dehydrogenase leakage. The integrity of the synaptosomal membrane was analyzed by monitoring the activity of the cytoplasmic enzyme lactate dehydrogenase (LDH) in the incubation medium. LDH activity was measured spectrophotometrically, by using the Enzyline LDH/HBDH Optimisé Unitaire kit, which follows the rate of conversion of NADH to NAD^+ at 340 nm. The absorbance was recorded at different time intervals and enzymatic activity was calculated. One unit of enzymatic activity (U) was defined as the product of oxidation of 1 μmole of NADH/min at room temperature. Total lysis, corresponding to 100% of LDH activity on the sample, was determined after treatment with Triton X-100 (1%). Lactate dehydrogenase leakage was expressed as a percentage of the total LDH activity in the synaptosomes.

Assessment of adenine nucleotides. Aliquots (1 mg protein) of freshly prepared synaptosomal fractions were extracted with ice-cold 0.2 M perchloric acid and centrifuged at 15,800 × *g* for 5 min at 4°C. The resulting supernatants were neutralized with KOH/Tris (10/5 M), stored at −80°C and assayed for adenine nucleotides (ATP, ADP, and AMP) by separation via reverse-phase high performance liquid chromatography (HPLC), as described elsewhere (Stocchi *et al*., 1985). The results were expressed as nmol/mg protein, and the energy charge potential (ECP) was calculated following the equation:

$$ECP = \frac{ATP + 0.5 \times ADP}{ATP + ADP + AMP} \quad (1)$$

In vitro *(Na^+/K^+)ATPase activity assay.* The (Na^+/K^+)ATPase activity of freeze-thawed samples was determined by measuring the release of inorganic phosphate (P_i) associated with the hydrolysis of ATP (Silva and Gonçalves, 2003). Aliquots (100 μg protein) were incubated in 1 ml of reaction medium (128 mM NaCl, 5 mM KCl, 10 mM $MgCl_2$, 100 μM EGTA and 10 mM HEPES-Na, pH 7.4) and various concentrations of ouabain, a selective inhibitor of the (Na^+/K^+)ATPase. The reactions were initiated by adding ATP-Mg and stopped by dilution with ice-cold TCA (5% final concentration). The precipitated protein was discarded by centrifugation, after which the supernatant was collected and the inorganic phosphate content of the samples was quantified by colorimetric reaction with the molybdate reagent (5% ferrous sulfate and 1% ammonium molybdate prepared in 1 N H_2SO_4). The reactions were carried out for 5 min at room temperature. Simultaneously, a standard curve of KH_2PO_4 (0–85 μM) was prepared. The (Na^+/K^+)ATPase activity was taken as the difference between the amount of P_i produced during 5 min in the absence or in the presence of 5 mM ouabain. The dose–response curves of (Na^+/K^+)ATPase to ouabain were analyzed by nonlinear fitting with a sum of one to three terms of Equation 2, assuming the presence of one to three independent, non-interconvertible, saturable inhibitory processes, exhibiting different affinities for ouabain (Berrebi-Bertrand *et al.*, 1990):

$$(Na^+/K^+)ATPase\,Activity(\%\,of\,maximal) = \frac{V_{\max}}{10^{(I-K_I)}+1} \quad (2)$$

where V_{max} is the maximal velocity observed in the absence of ouabain, I is the decimal logarithm of ouabain concentration, and K_I is the decimal logarithm of the 50% inhibitory concentration (IC_{50}).

Data analysis. All the data obtained were treated statistically with the "Microcal Origin 6.0" computer program. The results are presented as means ± S.E. of the number of experiments indicated in Figures 1–4 and Tables 1–2 Statistical significance between the two groups was analyzed by the unpaired two-tailed Student's *t*-test.

RESULTS

Dietary administration of 3.60 g of $AlCl_3$ for a period of 120 days (0.03 g/day) was sufficient to produce a significant enrichment in aluminum of synaptosomes isolated from rat brain cortex, because the total aluminum content increased from 10.9 ± 1.2 in control rats to 17.5 ± 0.7 ng Al^{3+}/mg protein ($p < 0.01$) in $AlCl_3$-exposed rats during the experimental period. Measurement of the leakage of components from the cytoplasm into the surrounding extracellular medium has been widely accepted as a valid method to estimate cell viability based on the integrity of the cell membrane. Determination of leakage of the cytosolic lactate dehydrogenase (LDH) activity is one of the indicators of cell damage. Table 1 shows that LDH released from synaptosomes isolated from rat brain after dietary administration of 3.60 g of $AlCl_3$ for 120 days (0.03

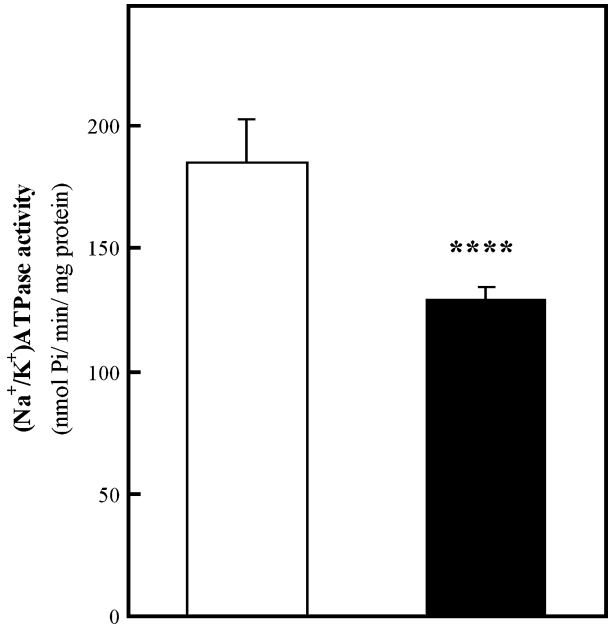

FIG. 1. Inhibition of synaptosomal (Na^+/K^+)ATPase activity by chronic exposure to aluminum. Freeze-thawed synaptosomes (0.1 mg protein/ml) were incubated during 5 min, at 35°C, in a medium containing 128 mM NaCl, 5 mM KCl, 10 mM $MgCl_2$, 100 μM EGTA, and 10 mM HEPES-Na (pH 7.4), in the absence and in the presence of 5 mM ouabain. The reactions were started by adding Mg-ATP to obtain final concentrations of 1 mM. After 5 min, the reactions were stopped and the (Na^+/K^+)ATPase activity was determined as described in the text. □, Control conditions (synaptosomes isolated from control rats); and ■, *in vivo* exposure to $AlCl_3$ (synaptosomes isolated from rats that received 0.03 g/day of $AlCl_3$ during 4 months). Data are the mean ± S.E. of six independent determinations. Statistical significance ****$p < 0.001$, compared to the control.

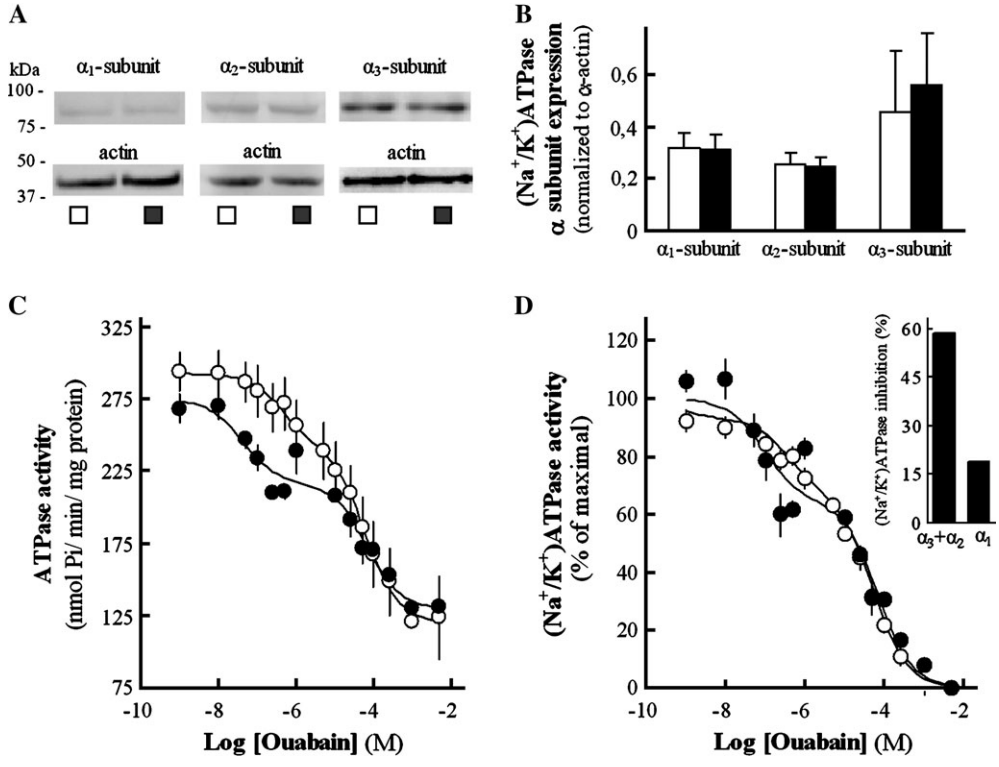

FIG. 2. Effect of aluminum exposure on endogenously expressed synaptosomal (Na^+/K^+)ATPase and on isozyme activities. At the end of the experimental period (120 days), synaptosomal fractions were prepared from brain cortex of control rats (□) or rats that received 0.03 g/day of $AlCl_3$ (■). Panel A. synaptosomal proteins (100 μg/lane) were separated by SDS-PAGE and subsequently immunoblotted with the anti-NASE (α_1-subunit), anti- HERED (α_2-subunit), anti-TED (α_3-subunit), and actin (α-actin) antibodies, as described in the text. Data shown are typical for seven independent experiments. Panel B. densitometric analysis of the Western blots. Panel C. Dose–response curves for the ouabain inhibition of ATPase activity. Experimental conditions are similar to those described in the legend of Figure 1, except that reactions were performed in the absence and in the presence of increasing ouabain concentrations (0–0.001 M). Data are expressed as nmol Pi/min/mg protein, and are the mean ± S.E. of 9–18 independent determinations. Panel D. Normalized dose–response curves for the ouabain inhibition of the (Na^+/K^+)ATPase activity of experimental data plotted in Panel C and expressed as a percentage of maximal activity in control conditions (○) and *in vivo* exposure to $AlCl_3$ (●), respectively. The curves represent the fitting by Equation 2, consisting of the sum of three functions assuming the presence of a very high affinity, a high affinity, and a low affinity, independent, non-interconvertible, saturable inhibitory processes that exhibit different affinities for ouabain, as described in the text. The insert represents the aluminum inhibitory effect on the (Na^+/K^+)ATPase activity in the presence of 10^{-6} and $5*10^{-3}$ M ouabain, because 10^{-6} M ouabain mostly inhibits the α_2- and α_3-isoforms, whereas the α_1-isoform remains largely active.

g/day) was comparable to the LDH released from synaptosomes isolated from control rats, indicating that chronic exposure to aluminum failed to produce significant damage of synaptosomal membranes. It should be noted that the activity of the cytosolic marker measured in lysed synaptosomes was 0.018 U/mg protein, in synaptosomes isolated from brain cortex of either control rats or $AlCl_3$-treated rats. Synaptosomal susceptibility was also evaluated on both synaptosomal preparations by the analysis of intrasynaptosomal adenine nucleotides. Adenine nucleotides provide ready-to-use substrates for carrying out normal cellular activities, and their levels are maintained by a balance between their production and degradation by specific enzymatic reactions. The levels of ATP/ADP and energy charge potential (ECP), which reflects the molar fraction of high-energy bond (Hardie and Hawley, 2001), were thereby analyzed to provide information on the metabolic status of nerve terminals after exposure to dietary aluminum. Upon exposure to aluminum, this balance was not significantly perturbed, and both ATP/ADP and ECP remained unchanged (Table 2). In fact, we did not observe significant changes in the amount of ATP (from 4.91 ± 0.15 to 5.03 ± 0.48 nmol/mg protein), ADP (from 1.49 ± 0.27 to 2.07 ± 0.43 nmol/mg protein) or AMP (from 4.56 ± 0.03 to 4.03 ± 0.50 nmol/mg protein). ATP represented 44.8% and 45.2% of the total adenine nucleotide content of synaptosomes isolated from control and aluminum-exposed rats, respectively.

As shown in Figure 1, in the presence of 1 mM Mg-ATP, which corresponds to a substrate-saturating concentration, the (Na^+/K^+)ATPase [ATP phosphohydrolase $(Na^+/K^+$-exchanging) (EC 3.6.3.9)] activity of freeze-thawed synaptosomes isolated from brain cortex of $AlCl_3$-treated rats was significantly reduced (30%) when compared to the activity exhibited by freeze-thawed synaptosomes isolated from brain cortex of control rats. Since previous results demonstrated that $AlCl_3$ concentrations up to 100 μM in the reaction medium does not change the ouabain-binding affinity of the (Na^+/K^+)ATPase

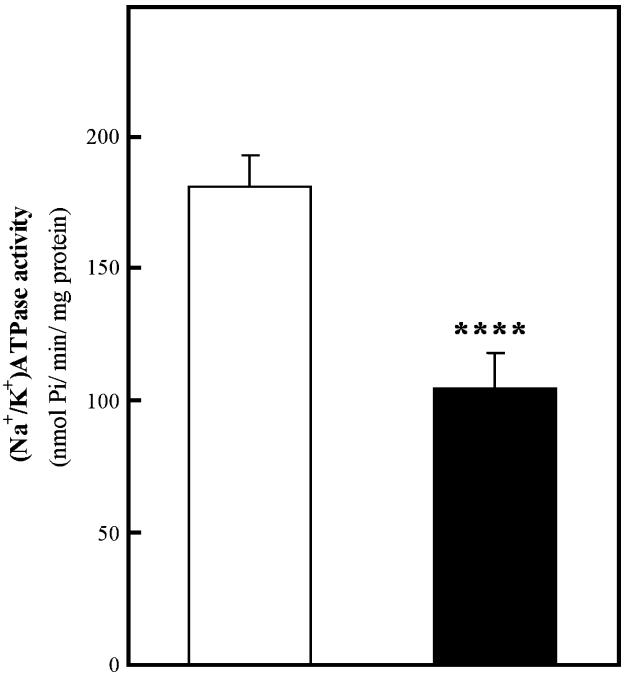

FIG. 3. Inhibition of kidney (Na^+/K^+)ATPase activity by chronic exposure to aluminum. Tissue homogenates prepared from kidney of control rats (0.1 mg protein/ml) were incubated for 5 min at 35°C, in a medium containing 128 mM NaCl, 5 mM KCl, 10 mM $MgCl_2$, 100 μM EGTA, and 10 mM HEPES-Na (pH 7.4), in the absence and in the presence of 5 mM ouabain. The reactions were started by adding Mg-ATP to obtain final concentrations of 3 mM. After 5 min, the reactions were stopped, and the (Na^+/K^+)ATPase activity was determined as described in the text. □, Control conditions (synaptosomes isolated from control rats); and ■, *in vivo* exposure to $AlCl_3$ (synaptosomes isolated from rats that received 0.03 g/day of $AlCl_3$ during 4 months). Data are the mean ± S.E. of 6 independent determinations. Statistical significance ****$p < 0.001$, compared to the control.

(Caspers *et al.*, 1994; Silva and Gonçalves, 2003), the enzyme activity was calculated as the difference between the adenosine triphosphatase (ATPase) activities in the absence and in the presence of 5 mM ouabain, the foremost utilized inhibitor to distinguish the (Na^+/K^+)ATPase among other phosphohydrolase activities. The data indicated that the onset of inhibition of

TABLE 1
Effect of $AlCl_3$ Exposure on Synaptosomal Membrane Integrity Measured by Lactate Dehydrogenase (LDH) Leakage

	LDH leakage (% of total)	Total LDH activity (U/mg protein)
Control	3.59 ± 0.45	0.018 ± 0.005
In vivo exposure to $AlCl_3$	4.18 ± 0.46	0.018 ± 0.006

Note. Analysis of LDH activity was performed in supernatants of synaptosomes isolated from control rats (control condition) and from rats that received 0.03 g/day of $AlCl_3$ during 4 months (*in vivo* exposure to $AlCl_3$) as described in the text. Data are the mean ± S.E. of 6 independent determinations.

TABLE 2
Effect of $AlCl_3$ Exposure on Synaptosomal Energy Adenine Nucleotides Balance

	ATP/ADP	ECP
Control	3.42 ± 0.73	0.52 ± 0.01
In vivo exposure to $AlCl_3$	2.82 ± 0.40	0.55 ± 0.01

Note. Quantification of adenine nucleotides was performed by HPLC in perchloric acid extracts of synaptosomes isolated from control rats (control condition) and from rats that received 0.03 g/day of $AlCl_3$ during 4 months (*in vivo* exposure to $AlCl_3$). Data are the mean ± S.E. of 2–6 independent determinations.

(Na^+/K^+)ATPase activity by aluminum (Fig. 1) appears to precede impairment of energy balance because ATP level was maintained at near control levels for the total period of aluminum exposure.

The (Na^+/K^+)ATPase is composed of stoichiometric amounts of two obligatory major polypeptides, the α-subunit (~112 kDa) and the β-subunit (~45 kDa). The binding sites for ATP, cations and ouabain are localized in the α-subunit, which is responsible for the catalytic activity of the enzyme. Three distinct isoforms of the α-subunit have been identified in the central nervous system of vertebrates (Mobasheri *et al.*, 2000; Sweadner, 1992). To confirm the expression of α_1, α_2, and α_3 subunits of (Na^+/K^+)ATPase in rat brain synaptosomal fraction (Foley and Linnoila, 1993), three antibodies against the catalytic subunit were used in the present study, namely anti-NASE, anti-HERED, and anti-TED. Western blot analysis revealed the presence of all the three α-subunit isoforms in the synaptosomes, because major immunoreactive bands of the three specific antibodies with relative molecular weights of 93 kDa (α_1-subunit), 92 kDa (α_2-subunit), and 98 kDa (α_3-subunit) were detected (Fig. 2A), although α_3-subunit was shown to be highly expressed (Fig. 2A and B). The (Na^+/K^+)ATPase α_1-, α_2-, and α_3-subunits are known to have different sensitivities to ouabain (Blanco and Mercer, 1998). Taking that into account, the synaptosomal isoform pattern was also checked by measuring the ATP phosphohydrolase dependence on the concentration of ouabain presented in the reaction medium (Fig. 2C). The dose–response curves for ouabain inhibition of membrane-bound (Na^+/K^+)ATPase activity exhibited the typical profile, currently interpreted as reflecting the presence of three (Na^+/K^+)ATPase α-subunit isoforms with distinct sensitivities to ouabain in brain tissue. To discriminate the ouabain sensitivities of the isozymes presented in the synaptosomal preparation, the experimental data were fitted to a curve described by an equation consisting of the sum of three functions assuming the presence of a very high, a high, and a low affinity, independent, non-interconvertible, saturable inhibitory processes exhibiting different affinities for ouabain (Fig. 2D). Since the experimental data were close to those obtained by nonlinear fitting of the titration curves, this approach was used to

calculate the 50% inhibitory concentration (IC_{50}) for ouabain of the hydrolytic reaction carried out by synaptosomal (Na^+/K^+)ATPase. We verified that the enzyme activity could be depicted in a very-high-sensitive ($IC_{50} = 5.4*10^{-10}$ M), a high-sensitive ($IC_{50} = 3.7*10^{-7}$ M), and a low-sensitive ($IC_{50} = 5.2*10^{-5}$ M) component. These values were consistent with previously reported ouabain sensitivity of rat brain (Na^+/K^+)ATPase α-subunit isoforms corresponding to 10^{-10}–10^{-7} M (α_3 −subunit), 10^{-7}–10^{-6} M (α_2 −subunit), and 10^{-5}–10^{-3} M (α_1−subunit) (Berrebi-Bertrand *et al.*, 1990; Gerbi and Maixent, 1999; Lopez *et al.*, 2002). In the presence of Na^+ (128 mM), K^+ (5 mM), Mg^{2+} (10 mM), and saturating concentrations of ATP, the very high, high, and low ouabain sensitive components contributed to approximately 6%, 26%, and 68% of total (Na^+/K^+)ATPase activity.

To determine whether dietary administration of 3.60 g of $AlCl_3$ for 120 days (0.03 g/day) caused significant changes in the protein expression levels of α-subunit isoforms of synaptosomal (Na^+/K^+)ATPase, analysis of NASE (α_1), HERED (α_2), and TED (α_3) immunopositive bands normalized to α-actin, were performed in control and chronic dietary aluminum–exposed rats (Fig. 2A and B). No significant changes in protein expression of the three α-subunit isoforms were observed in synaptosomes from control and $AlCl_3$-treated rats ($p > 0.7$ for all). Accordingly, the dose–response curves for ouabain inhibition of membrane-bound (Na^+/K^+)ATPase activity remained almost unchanged upon exposure to $AlCl_3$ (Fig. 2C), exhibiting values for IC_{50} of $4.5*10^{-8}$, $1.1*10^{-7}$, and $6.5*10^{-5}$ M (Fig. 2D). These results seemed to indicate that *in vivo* exposure to $AlCl_3$ inhibits synaptosomal (Na^+/K^+) ATPase activity without compromising either the expression of α-subunit isoforms or the ouabain sensitivity (high and low) of the isozymes. Interestingly, *in vivo* exposure to $AlCl_3$ appears to render the enzyme less responsive to ouabain in the range of very low concentrations. To estimate the inhibitory effect of aluminum on the distinct isozymes, the synaptosomal ATPase activities in the absence and in the presence of 10^{-6} and $5*10^{-3}$ M ouabain were contrasted, because 10^{-6} M ouabain mostly inhibits α_2- and α_3-isoforms, whereas the α_1-isoform remains largely active. It was observed that both components (α_2/α_3-isoforms and the α_1-isoform) of the total enzyme activity contributed to the overall decrease (30%) in (Na^+/K^+)ATPase activity induced by aluminum, given that α_2/α_3, and α_1 components were reduced by 59% and 19%, respectively, when compared to the activities exhibited by freeze-thawed synaptosomes isolated from brain cortex of control rats (Fig. 2D, insert).

It is generally accepted that in rat kidney only α_1-subunit and β_1-subunit isoforms are present (Mobasheri *et al.*, 2000), which corresponds to α_1-β_1 protomer of (Na^+/K^+)ATPase that is highly resistant to inhibition by ouabain ($IC_{50} = 10^{-5}$–10^{-3}M) (Dmitrieva and Doris, 2003; Gerbi and Maixent, 1999; Lopez *et al.*, 2002). Nonetheless, a slight inhibition of kidney (Na^+/K^+)ATPase activity by ouabain at submicromolar concentration has been reported (Dmitrieva and Doris, 2003). To confirm the effect of the *in vivo* exposure to $AlCl_3$ on (Na^+/K^+)ATPase, the enzyme activity (Fig. 3) and subunit protein expression (Fig. 4) were also assessed in kidney, where the aluminum increment during the 4 months of exposure was 3.5 ng Al^{3+}/mg protein.

In our experimental conditions, only the α_1-subunit of the (Na^+/K^+)ATPase was found in kidney homogenates (Fig. 4A). In accordance with data obtained in brain synaptosomes, a decrease in (Na^+/K^+)ATPase activity was observed in kidney homogenates after chronic exposure to aluminum (Fig. 3). Nevertheless, no significant changes in (Na^+/K^+)ATPase α_1-subunit expression were observed in the $AlCl_3$-treated group, compared to the control (Fig. 4B). It is interesting to note that the isoform pattern in $AlCl_3$-exposed rat heart and brain total homogenates, in which the α_1-, α_2-, and α_3-subunits are present, remained unchanged, and no significant increment in aluminum content was observed (data not shown). Conversely, the (Na^+/K^+)ATPase activity was significantly reduced (42%) after *in vivo* exposure to $AlCl_3$ (Fig. 3). The dose–response curves for ouabain inhibition of the enzyme activity overlapped in the range of high concentrations ($5*10^{-5}$ M) of ouabain (Fig. 4C) and the IC_{50} remained almost unchanged during exposure to $AlCl_3$, exhibiting values of $6.1*10^{-5}$ M (control conditions) and $3.7*10^{-4}$ M (*in vivo* exposure) (Fig. 4D). In contrast, *in vivo* exposure to $AlCl_3$ completely abolished the inhibition of (Na^+/K^+)ATPase activity by ouabain in the range of concentrations below 10^{-6} M.

These results strongly suggest that aluminum accumulation in nerve terminals during chronic dietary $AlCl_3$ administration decreases the (Na^+/K^+)ATPase activity without compromising the expression of α-subunit isoforms, and it does this before any significant damage to the synaptic plasma membrane or perturbation of energy levels occurs.

DISCUSSION

The cause of (Na^+/K^+)ATPase inhibition by aluminum remains controversial (Caspers *et al.*, 1994; King *et al.*, 1983; Lai *et al.*, 1980; Lal *et al.*, 1993; Rao, 1992; Sarin *et al.*, 1997; Silva and Gonçalves, 2003). Some authors have proposed stabilization of the phosphorylated form of the enzyme and changes in the structure of plasma membrane (Caspers *et al.*, 1994; Lal *et al.*, 1993; Rao, 1992; Sarin *et al.*, 1997). Others have suggested that failure of the hydrolytic activity results from concurrent ATP depletion as a result of mitochondrial impairment and/or formation of non-hydrolysable Al-ATP complexes (Kohila *et al.*, 2004; Panchalingam *et al.*, 1991). Aluminum appears to act as a noncompetitive inhibitor of the ATP hydrolysis by the (Na^+/K^+)ATPase (Caspers *et al.*, 1994; Silva and Gonçalves, 2003), and total inhibition has never been reported in the range of micromolar concentrations. The functional activity of (Na^+/K^+)ATPase in

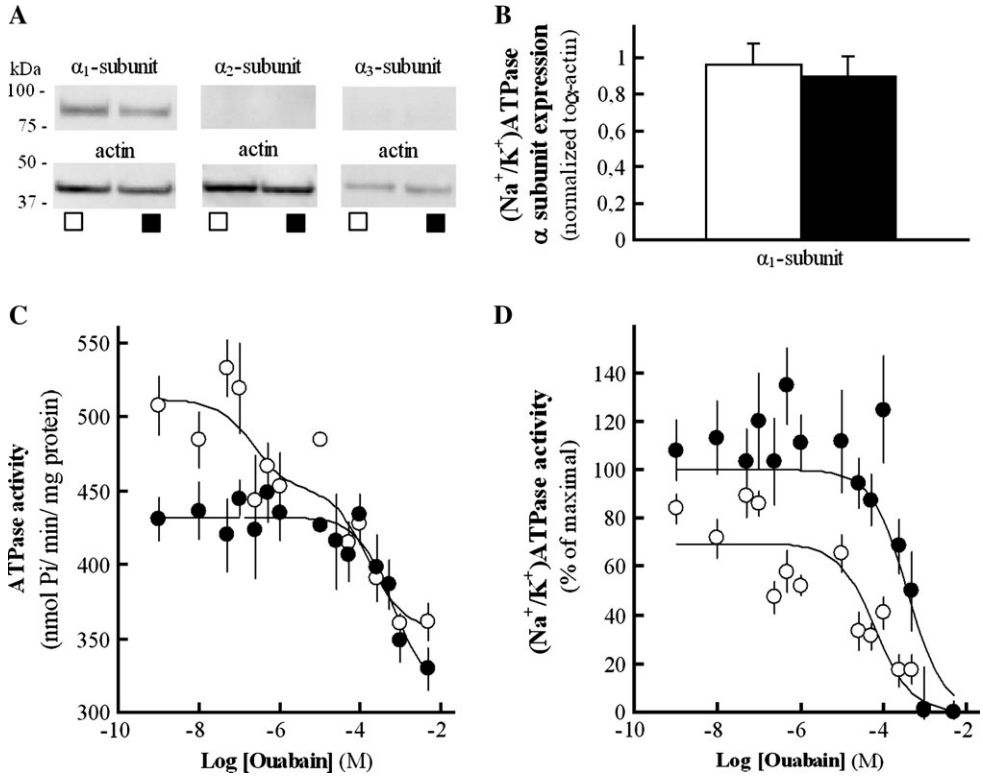

FIG. 4. Effect of aluminum exposure on endogenously expressed kidney (Na^+/K^+)ATPase and on isozyme activity. At the end of the experimental period (120 days), tissue homogenates were prepared from kidney of control rats (□) and rats that received 0.03 g/day of $AlCl_3$ (■). Panel A. kidney proteins (100 μg/lane) were separated by SDS-PAGE and subsequently immunoblotted with anti-NASE (α_1-subunit), anti- HERED (α_2-subunit), anti-TED (α_3-subunit), and actin (α-actin) antibodies, as described in the text. Data shown are typical for seven independent experiments. Panel B. Densitometric analysis of the Western blots. Panel C. Dose–response curves for the ouabain inhibition of the ATPase activity. Experimental conditions are similar to those described in the legend of Figure 3, except that reactions were performed in the absence and in the presence of increasing ouabain concentrations (0–0.001 M). Data are expressed as nmol Pi/min/mg protein, and are the mean ± S.E. of 8–16 independent determinations. Panel D. Normalized dose–response curves for the ouabain inhibition of the (Na^+/K^+)ATPase activity of experimental data plotted in Panel C and expressed as a percentage of maximal activity in control conditions (○) and *in vivo* exposure to $AlCl_3$ (●), respectively. The curves represent the fitting by Equation 2, assuming the presence of one saturable inhibitory process by ouabain.

different tissues and cell types is closely related to the existence of multiple isozymes consisting of different subunit isoforms and displaying different kinetic properties (see Blanco and Mercer, 1998; Cornelius and Mahmmoud, 2003; Jorgensen *et al.*, 2003; Kaplan, 2002; Mobasheri *et al.*, 2000, for recent reviews).

We demonstrated that the protein expression levels of the (Na^+/K^+)ATPase catalytic subunit is not diminished in rat kidney, heart, or brain after chronic exposure to dietary aluminum (daily supply of 0.03 g of $AlCl_3$ during 4 months). The quantitative analysis of ouabain dose–response curves and Western blotting analysis of immunopositive bands using antibodies directed against specific amino acid sequences of the α_1-, α_2-, and α_3-subunit isoforms of the (Na^+/K^+)ATPase (Figs. 2A and 2B and 4A and 4B) revealed that aluminum exposure did not significantly modify both the relative expression level of each catalytic subunit isoform and the characteristic isoform expression profile of the examined tissues. The decrease of expression level of one of the (Na^+/K^+)ATPase α-subunit isoforms is usually associated with a shift from one isoform to another or accompanied by a parallel decrease of all isoform levels, despite each isoform being encoded by a different gene (Charlemagne *et al.*, 1994; Serluca *et al.*, 2001).

Comparison of the α-subunit isoform expression profile did not reveal any insightful differences that accounted for the observed differences in (Na^+/K^+)ATPase activity (Figs. 1 and 3). Therefore, this is the first experimental demonstration that an aluminum-induced decrease of maximal enzyme activity cannot be attributed to the reduction of the expression levels of the catalytic subunits. These results, combined with outstanding similarity of *in vivo* and *in vitro* aluminum effects on the enzyme activity previously reported by Silva and Gonçalves (2003), support the hypothesis that aluminum either acts on post-transcriptional regulatory mechanisms or directly interacts with the enzyme. Under our experimental conditions, aluminum inhibited all three α-subunit isoforms of the (Na^+/K^+)ATPase, which does not support FXYD protein-mediated inhibitory action of aluminum. The FXYD regulatory subunits act in a tissue- and αβ-protomer–specific manner, modifying the Na^+ and/or K^+ affinities of the enzyme (Cornelius and

Mahmmoud, 2003). This assumption is also indicated by a lack of aluminum-induced changes of enzyme activation by Na^+ (Caspers *et al.*, 1994). According to previous observations, aluminum does not appear to interact, directly or indirectly, on Na^+, K^+ and high-affinity ATP binding sites of the (Na^+/K^+)ATPase, which makes it improbable that the noncovalent αβ-protomer will reassemble during *in vivo* exposure to $AlCl_3$. Typically, different α-β–subunit combinations with the same catalytic subunit isoform differ with regard to control of biosynthesis and properties, such as Na^+, K^+ and ATP affinities (Geering, 2001). Furthermore, the binding sites for ATP and cations are localized in the α-subunit and only the αβ-protomers are able to carry out (Na^+/K^+)ATPase activity. The reduction by $AlCl_3$ of ATPase activity under conditions that favor maximal activation of the (Na^+/K^+)ATPase reported here (Figs. 1 and 3) has been consistently observed by other investigators (Caspers *et al.*, 1994; King *et al.*, 1983; Lai *et al.*, 1980; Lal *et al.*, 1993; Rao, 1992; Sarin *et al.*, 1997), and it is difficult to reconcile with simple aluminum-induced dissociation of αβ-protomeric units, because the aluminum inhibitory effect requires the presence of high ATP concentrations and does not occur when uridine triphosphate (UTP), a poor ATP substitute at low-affinity nucleotide binding sites, is used as a hydrolytic substrate (Silva and Gonçalves, 2003).

As evidenced from our data, aluminum-induced inhibition of (Na^+/K^+)ATPase activity is resistant to cell fractionation (isolation of synaptosomes) and freeze-thaw procedures, suggesting that it remains trapped on the membrane fraction. It is interesting to note that when assessed by *in vitro* approaches, much higher aluminum chloride concentrations were required (at micromolar range), and the extent of the inhibition appeared to be time-dependent (Caspers *et al.*, 1994; Lai *et al.*, 1980). Taking together, these results seem to implicate high-affinity and low accessibility of aluminum binding sites that are responsible for the inhibition of (Na^+/K^+)ATPase. In fact, it was previously suggested that after *in vivo* aluminum exposure, substantial amounts of aluminum remain bound to high molecular weight proteins (Julka *et al.*, 1996). Although the results presented in this work do not define the mechanism responsible for $AlCl_3$-induced alteration of (Na^+/K^+)ATPase activity, they are in agreement with the hypothesis that aluminum induces impairment of the interprotomeric interaction within the oligomeric ensemble of membrane-bound (Na^+/K^+)ATPase (Silva and Gonçalves, 2003). It remains to be clarified whether aluminum interacts directly with the subunits of the enzyme or interferes indirectly with the oligomerization of the membrane-bound enzyme's protomeric functional units of the (Na^+/K^+)ATPase, after aluminium-induced alterations of membrane fluidity, enhancement of free radical production, and impairment of the phosphorylation cascade (Boldyrev, 2001; Laughery *et al*., 2004).

The present study provides data related to the analysis of membrane integrity (Table 1) and energy status (Table 2) of synaptic nerve terminals obtained from rat brains chronically exposed to aluminum. Because no significant changes were observed in LDH leakage, ECP values, or ATP/ADP levels, the results are consistent with the idea that these parameters reflect the maintenance of cell viability (Hardie and Hawley, 2001). Moreover, an adequate energy supply seems to be assured during the aluminum exposure protocol reported in this work, because the overall high-energy phosphate content, reflected by the ECP, was maintained. In fact we may consider that, inhibition of (Na^+/K^+)ATPase (the major energy consumer in a cell) would be beneficial for preserving high intracellular ATP level. It is increasingly recognized that, depending on intensity/duration parameters of the insult, aluminum can produce cell death either by apoptosis or by necrosis (Johnson *et al.*, 2005), and maintenance of ATP is required for apoptotic cell death pathways (Kim *et al.*, 2003). Accordingly, Savory *et al*. (2003) demonstrated that agents interfering with the mitochondrial and/or the endoplasmic reticulum–mediated apoptosis cascade have the ability to prevent aluminum-induced apoptosis in rabbit brain, which is in line with the idea that modifications in the intracellular calcium homeostasis and potentiation of transition metal pro-oxidant action could mediate toxic effects during chronic aluminum exposure. Moreover, Julka and Gill (1996) observed that exogenous addition of glutathione, an endogenous antioxidant, could only partially reverse Ca^{2+}-ATPase inhibition after *in vivo* administration of aluminium, whereas desferrioxamine, an aluminum chelator, produced total reversion. Thus inhibition of (Na^+/K^+)ATPase could be interpreted as a way the cells signal leads to eventual cell death through apoptosis (Yu, 2003).

Apparently, *in vivo* aluminum-induced partial inhibition of (Na^+/K^+)ATPase activity should promote a continuous reduced enzyme activity irrespective of subtle differences in adapting cellular enzyme activity to specific physiological requirements. Instead, *in vivo* exposure to $AlCl_3$ seemed to specifically reduce the (Na^+/K^+)ATPase sensitivity to ouabain at submicromolar concentrations (Figs. 2C and 4C), and to prevent the stimulatory effect produced by ATP binding with low affinity (Silva and Gonçalves, 2003). According to several investigators, both features—the ouabain very highly sensitive inhibition and the stimulatory ATP effect—are even more pronounced when the membrane-bound (Na^+/K^+)ATPase exhibits high levels of oligomerization (Boldyrev, 2001; Laughery *et al.*, 2004). Moreover, high-affinity cardiotonic steroid binding capacity of the enzyme is relevant for (Na^+/K^+)ATPase–mediated signal transduction within caveolae (Xie and Cai, 2003). Chemical cross-linking between neighbouring active α-β protomers (oligomerization) is a specific process, giving rise to the formation of complexes highly sensitive to changes in microviscosity and signaling molecules (Boldyrev, 2001). The presence of (Na^+/K^+)ATPase within caveolae provides a means of explaining the importance of multimeric interactions between active protomeric ensembles, since these membrane morphological and functional units are involved in signaling pathways, starting from the plasma membrane. It is

clear that inhibition of the (Na^+/K^+)ATPase leads, in a cell-specific manner, to activation of multiple signaling pathways (*i.e.*, MEK/MAPK-ERK and Src family kinase pathways), to endoplasmic reticulum inositol 1,4,5-trisphosphate receptor-mediated intracellular Ca^{2+} oscillations and to mitochondrial production of reactive oxygen species (see Xie and Cai, 2003 for review).

In conclusion, the present study shows that during aluminum exposure, inhibition of (Na^+/K^+)ATPase activity occurs, preceding possible alterations of expression of catalytic subunits, cellular energy depletion, and disturbances in cellular membrane integrity. The decrease in total (Na^+/K^+)ATPase activity is ensured by partial inhibition of isozymes containing α_1-, α_2-, and α_3-subunits. In addition, aluminum-induced inhibition of the enzyme activity was maintained post-exposure; therefore the preparative procedures and activity assays might have contributed to underestimates of the degree of inhibition of (Na^+/K^+)ATPase in *in vivo* aluminum-exposed rats. These data also support the concept that (Na^+/K^+)ATPase inhibition occurs at early stages of the neurotoxic action, because no evidence of cell death/membrane disruption or energy depletion were observed in synaptosomes isolated from $AlCl_3$ chronically exposed rats.

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